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Little evidence that Beach Pea (*Lathyrus japonicus*) toxins cause gait abnormalities in Sable Island Feral Horse (*Equus ferus caballus*)

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Abstract
Lathyrus is a leguminous plant genus notable for its synthesis of lathyrogens, a group of nonprotein amino acids. Lathyrogens can have a range of toxic effects on mammals when consumed in large quantities, but research on lathyrogens has occurred primarily in an agricultural context and on the syndromes (osteo-, angio-, and neuro-lathyrism) that *Lathyrus* consumption causes in humans and livestock. We know relatively little about whether the lathyrogens in uncultivated *Lathyrus* species are more broadly consequential to the ecology of natural ecosystems. For example, the unmanaged Feral Horse (*Equus ferus caballus*) population inhabiting Sable Island, Nova Scotia, Canada relies on forage from coastal maritime plant communities, including Beach Pea (*Lathyrus japonicus*). Horses have a strong sensitivity to lathyrogens compared with many other mammals, and symptoms of lathyrism (gait abnormalities, hernias, hoof malformation) are sometimes observed in Sable Island horses. However, it is unclear whether Beach Pea on Sable Island produces lathyrogens at sufficient concentrations to cause disease. Using liquid chromatography–mass spectrometry (LC-MS), we tested immature, mature, and senescent Sable Island Beach Pea samples for two of the most common lathyrogens: L-β-N-oxalyl-α,β-diaminopropionic acid (β-L-ODAP) and β-aminopropionitrile (BAPN). We detected only trace amounts of β-L-ODAP and no BAPN, causing us to conclude that lathyrogens are unlikely to be the cause of the physical abnormalities observed in Sable Island horses. These results from an iconic Canadian ecosystem provide useful toxicological data for a common coastal maritime plant.

Key words: Lathyrogen; lathyrism; liquid chromatography–mass spectrometry; ODAP; BAPN; DABA; equine; zoopharmacognosy; Beach Pea; *Lathyrus japonicus*; Sable Island; horse; *Equus ferus caballus*

Introduction
Species of the leguminous genus *Lathyrus* L. (Fabaceae) are notable for producing toxins (lathyrogens) that cause a range of eponymous diseases in mammalian herbivores when consumed in large quantities (Stunkard 1974). One of these, neurolathyrism, is among the earliest recorded diseases in humans, dating to writings by Hippocrates (Dastur and Iyer 1959). Neurolathyrism is caused by L-β-N-oxalyl-α,β-diaminopropionic acid (β-L-ODAP), a nonprotein amino acid found in most *Lathyrus* species, most notably Grass Pea (*Lathyrus sativus* L.), a staple crop in some parts of the world (Manna *et al.* 1999). In people affected by malnutrition, prolonged consumption of β-L-ODAP can cause motor neuron degeneration in the distal corticospinal tract (Spencer *et al.* 1986). This results in a stiff or spastic gait and, in severe cases, leg and arm contractures, irreversible atrophy of leg muscles, and spastic paraparesis (Rao *et al.* 1967; Stunkard 1974; Misra *et al.* 1993).

Other species of *Lathyrus* have been found to produce different toxic nonprotein amino acids. For example, Sweet Pea (*Lathyrus odoratus* L.) synthesizes β-aminopropionitrile (BAPN; Wiley and Joneja 1976). Unlike β-L-ODAP, BAPN interferes with collagen formation (Brüel *et al.* 1998). In doing so, BAPN causes osteolathyrism and angiolathyrism, diseases characterized by bone deformation, hernia formation, and increased risk of aortic rupture (Strong 1966; Conner and Peacock 1973).

Most *Lathyrus* species tested have been found to contain β-L-ODAP or BAPN (Ghosh *et al.* 2015);

however, testing efforts have been heavily biased toward *Lathyrus* species used in agriculture or as ornamentals. As a result, we have little understanding of how the consumption of lathyrogens affects wild animal populations.

Beach Pea (*Lathyrus japonicus* Willdenow) is an uncultivated *Lathyrus* species that has a circumpolar distribution and is found predominately in temperate coastal environments (Lechowicz *et al.* 1980). Although Beach Pea is not a cultivated crop, its colourful flowers and large pea pod (legume) fruits are sometimes harvested for food on a non-commercial basis (Chinnasamy *et al.* 2004; Egebjerg *et al.* 2018). In the non-peer-reviewed recreational foraging literature, there are conflicting reports as to whether Beach Pea is safe to consume, or if, like other *Lathyrus* species, it is toxic (Turner and von Aderkas 2009; Seymour 2020). Chavan *et al.* (2003) detected small amounts of β-L-ODAP in the seeds of Beach Pea samples collected from Bellevue Beach, Newfoundland and Labrador, Canada, but none in Beach Pea leaves or pods. However, in Grass Pea, β-L-ODAP concentrations can differ with genotype (Kumar *et al.* 2011), abiotic conditions, or developmental stage (Jiao *et al.* 2011). Therefore, it is unclear whether the observations made by Chavan *et al.* (2003) near the northern extent of the Beach Pea's range and at a single developmental stage are broadly representative of the ability of Beach Pea to synthesize lathyrogens. Similarly, it is unclear whether Beach Pea plants might instead produce other lathyrogens (e.g., BAPN) at greater concentrations than β-L-ODAP.

We have recorded gait abnormalities (upward fixation of the patella) in Sable Island Feral Horse (*Equus ferus caballus*; estimated rates and age distribution of this condition in the population not currently available although observed in both mature and immature horses), which we hypothesized might be symptomatic of neurolathyrism (see Video S1). Furthermore, hernia (Figure 1a) and hoof malformations (Figure 1b) are observed frequently enough that the size, shape, and placement of these abnormalities can be used as diagnostic features to identify individual horses on Sable Island (~3% and 25% of the population, respectively; Welsh 1975; Mellish *et al.* 2021). Both hoof malformations and hernias could be symptoms of the collagen-disrupting effects of BAPN. However, gait abnormalities can also be caused by poor conformation or poor quadriceps muscle tone (Jeffcott and Kold 1982; Andersen and Tnibar 2016). Similarly, hoof malformation and hernias could be caused by environmental or nutritional deficiencies (Verschooten *et al.* 1994; Pollitt 2004). Determining whether biologically relevant levels of lathyrogens are present in Beach Pea plants on Sable Island is useful in testing competing hypotheses about the mechanisms underlying abnormal trait variation observed in Sable Island Feral Horses.

Mammalian species differ in their resistance to lathyrogens. Ruminants are relatively tolerant compared with humans and hindgut fermenters (Hanbury *et al.* 2000), and horses are highly sensitive to *Lathyrus* consumption (Holbrook *et al.* 2015). In horses, neurolathyrism is typically characterized by dyspnoea, as well as gait abnormalities caused by the stiffening and eventual paralysis of the hindlimbs (Enneking 2011). However, the clinical presentation of BAPN poisoning in horses is unknown, because the most well-studied BAPN-producing *Lathyrus* (Sweet Pea) is an ornamental species (Bao *et al.*

FIGURE 1. Representative cases of a. umbilical hernia and b. hoof malformation commonly observed in Feral Horse (*Equus ferus caballus*) on Sable Island, Nova Scotia, Canada. Photo a: Mason R. Stothart. Photo b: Alice Liboiron.

2020). Here, we sought to test whether gait and physiological (e.g., hoof deformation, hernias) abnormalities observed in Sable Island horses might be caused by lathyrogens, by quantifying β-ODAP and BAPN concentrations in immature and mature Beach Pea plants from Sable Island. If detected, the consumption of Beach Pea plants by Sable Island horses could present a useful opportunity to study the ecology of lathyrogen production and detoxification in nature.

Methods

Sable Island study system

Horses were first introduced to Sable Island, Nova Scotia, Canada (43.932°N, 59.938°W) in the mid-1700s, and they have since remained largely unmanaged, with legal protection after a 1961 amendment to what is now the *Canada Shipping Act (2001)* and, after 2013, under the *Canada National Parks Act*. Since 2007, this horse population has also been the focus of a long-term individual-based ecological study (Regan *et al.* 2020). American Beachgrass (*Calamagrostis breviligulata* (Fernald) Saarela subsp. *breviligulata* [formerly *Ammophila breviligulata* Fernald]) and various forbs, including such species as Seabeach Sandwort (*Honckenya peploides* (L.) Ehrhart) and Common Yarrow (*Achillea millefolium* L.), comprise most of the diet of horses on Sable Island (Welsh 1975). However, the horses also consume Beach Pea plants, which are found in locally high abundance across the island (Figure 2; Tissier *et al.* 2013).

Sample collection and preparation

In August 2020, samples of Beach Pea plants were collected opportunistically from five sites across Sable Island. Each site represented a patch of Beach Pea plants, generally intermixed with other vegetation, and all samples were collected within an area of ~10 m × 10 m. At each site, we collected samples of young shoots (new growth on small plants), flowering shoots, mature shoots with pods, and senescent shoots (with pods). These shoots were categorized as "young" (n = 5) or "old" (n = 5; bearing either inflorescence or fruit). Samples were stored at –20°C for several months, before being lyophilized and ground to a fine powder using a coffee grinder. Storage at –20°C preserves the stability of β-L-ODAP (Bento-Silva *et al.* 2019).

Determination of β-ODAP and BAPN by liquid chromatography–mass spectrometry (LC-MS)

For measurement of β-L-ODAP and BAPN, individual samples (25–50 mg of fine ground material) were extracted in 70% ethanol in three cycles (overnight, 1 h, 1 h) while shaking at room temperature and combined extracts were evaporated to dryness, followed by re-dissolving in 1 mL of dH_2O. This extraction protocol has been validated for β-L-ODAP recovery (Xiong *et al.* 2015; Emmrich *et al.* 2019), although others report reliable extractions can also be obtained using only dH_2O (Bento-Silva *et al.* 2019). Three replicate extractions were performed for each sample. We derivatized 20 µL of amino acids in a 100-µL reaction using Waters AccQ-Tag reagent (Waters, Milford, Massachusetts, USA) following the manufacturer's protocol. The derivatized samples were diluted 100-fold, and 5 µL samples were analyzed by LC-MS. The LC-MS estimates were normalized by starting sample mass.

Quantification was performed using a Xevo triple quadrupole TQ-S instrument (Waters) as previously described (Emmrich *et al.* 2019). The declustering potential, the collision energy, and the collision cell exit potential were optimized individually using standards and the automated method development tool (Intellistart) in the Waters MassLynx software. The test standards of β-L-ODAP (Lathyrus Technologies,

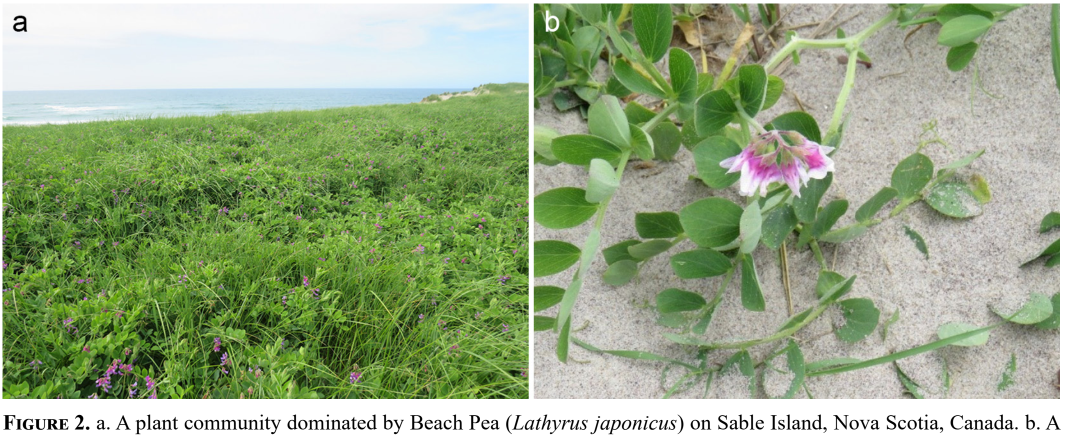

FIGURE 2. a. A plant community dominated by Beach Pea (*Lathyrus japonicus*) on Sable Island, Nova Scotia, Canada. b. A Beach Pea plant. Photos: Mason R. Stothart.

Hyderabad, India) were allowed to isomerize to create a mixture of α-L-ODAP and β-L-ODAP and BAPN (VWR International). The mass transitions used were 347.16 > 171.099 (ODAP) and 241.108 > 171.099 (BAPN).

Beach Pea extracts were spiked with standards to determine the limit of detection of ODAP and BAPN. To this end, 18 µL of young Beach Pea extract was mixed with 2 µL of 50 ng/µL α/β-L-ODAP or BAPN standards, in three technical replicates.

Results

Spiking of young Beach Pea samples with both α/β-L-ODAP and BAPN at a level of 5 ng/mL produced measurable responses, indicating a level of detection of 200 parts per billion (ppb; Figure S1, S2). Trace levels of β-L-ODAP (<10 parts per million [ppm]) were observed in both young and mature Beach Pea samples (Figure 3). However, no BAPN (at a level of detection of 200 ppb) was observed in any of the samples (Figure S3).

Discussion

We observed no evidence of BAPN and only trace amounts of β-L-ODAP in Beach Pea plants collected from Sable Island, regardless of stage of maturity. These results agree with previous findings reported in the literature for the species under a different name (*Lathyrus maritimus* Bigelow, a synonym of *L. japonicus*) and a different chemical name (β-N-oxalylamino-L-alanine, a synonym for β-L-ODAP; Chavan *et al.* 2003). Chavan *et al.* (2003) did not detect β-L-ODAP in the leaves or pods of Beach Pea samples collected from Bellevue Beach; however, they did observe trace amounts in Beach Pea branches (0.65 mg/100 g of dried material), fresh seeds (2.90

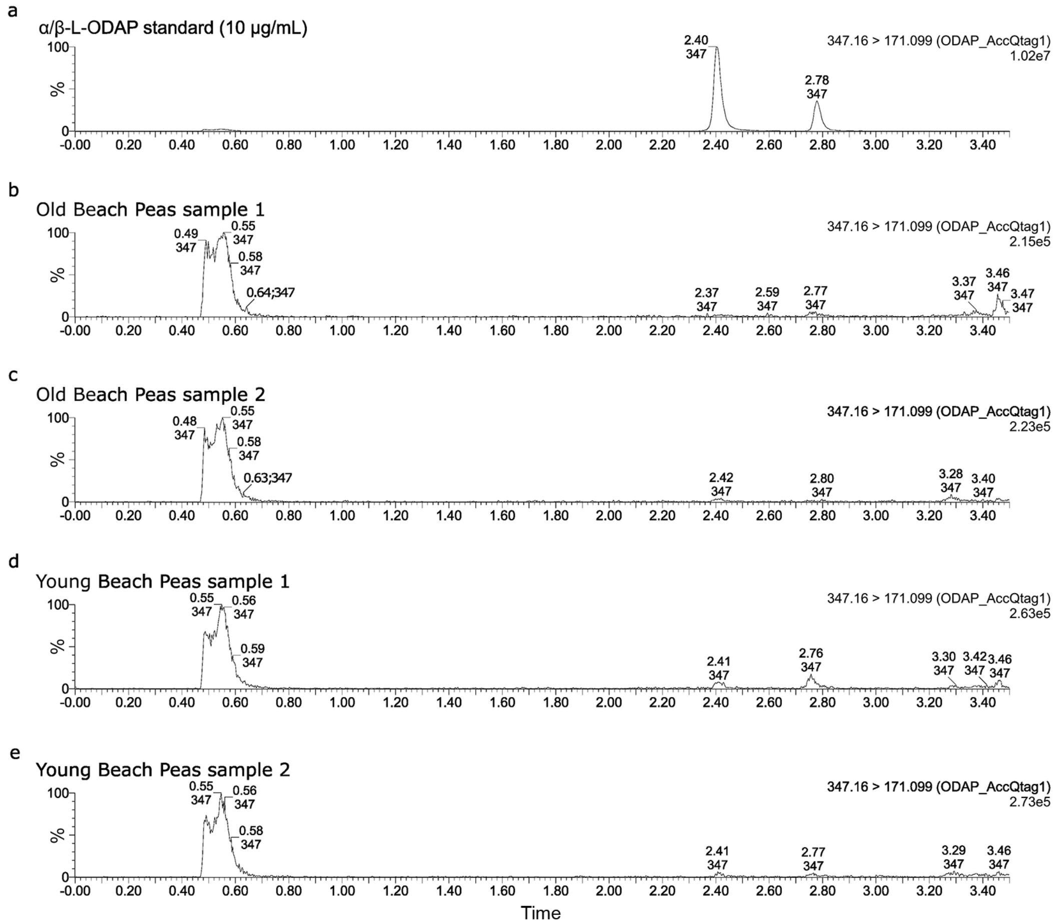

FIGURE 3. Detection of oxalyl-α,β-diaminopropionic acid (ODAP) in Beach Pea (*Lathyrus japonicus*) on Sable Island, Nova Scotia. For each age class, two liquid chromatography–mass spectrometry traces are shown (mass transition 347.16 > 171.099).

mg/100 g of dried material), and mature seeds (4.02 mg/100 g of dried material). In our samples, which comprised a homogenous mixture of leaf, stem, pod, and seeds, β-L-ODAP was only detectable because of the high sensitivity of the LC-MS method (Emmrich *et al.* 2019). The β-L-ODAP might have been present in higher concentrations in seeds of Sable Island Beach Pea plants; however, the leaf-stem-pod-seed mixtures that we assayed are more ecologically realistic approximations of how Beach Pea plants are consumed by the horses on Sable Island, which do not discriminate in terms of what parts of the plant they consume. Regardless, using either our estimates of β-L-ODAP in Beach Pea seeds or those made by Chavan *et al.* (2003), we expect the ingestion of lathyrogens by Sable Island horses to be negligible. Previous estimates of β-L-ODAP concentrations in Beach Pea plants are 10–50-fold lower than what is reported in the seeds of "low β-L-ODAP" cultivars of Grass Pea (Das *et al.* 2021). For comparison, Grass Pea seeds with β-L-ODAP-concentrations of ~20−2590 mg/100 g of material are widely consumed as human food and only pose a health risk if used as a staple food source during times of prolonged malnutrition.

Beach Pea plants on Sable Island do not appear to contain biologically significant concentrations of β-L-ODAP, but we cannot rule out the possibility of seasonal variation in lathyrogen content, because all samples were collected in August. Lathyrogen concentration can be higher in young than old plants (Jiao *et al.* 2011), and so, it is possible that β-L-ODAP concentrations may be greater in the early spring than in August. Similarly, environmental conditions, such as drought, salinity, toxic heavy metals, and symbioses with *Rhizobium* bacteria, could also affect β-L-ODAP content (Jiao *et al.* 2011).

Nonetheless, our results do not support the theory that Beach Pea consumption causes gait abnormalities, hernias, and hoof malformation in feral Sable Island horses. The gait abnormalities we see in Sable Island horses are also consistent with upper fixation of the patella (locking stifles). Locking stifles can be caused by deformations in femur, patella, or tibia, and poor tone in the quadriceps muscles, preventing patellar ligaments from being released from the medial trochlear ridge (Jeffcott and Kold 1982; Labens *et al.* 2005; Andersen and Tnibar 2016). These symptoms are consistent with osteolathyrism and neurolathyrism, respectively (Stunkard 1974), but they can also be caused by injury or have a genetic basis (Kidd 2017). Similarly, although the frequency of hernias and hoof malformation in this population could signal disrupted collagen formation, hernias in horses can be congenital or caused by blunt trauma or pregnancy and parturition (Moustafa *et al.* 2022) with many of the hernias we observe in horses on Sable Island appearing to be umbilical hernias. Meanwhile, hoof malformations can be linked to dietary imbalances or laminitis, which itself can be indicative of systemic disease (Verschooten *et al.* 1994; Pollitt 2004; Patterson-Kane *et al.* 2018). The sandy substrate of Sable Island may also contribute to hoof overgrowth, because horses do not have access to hard substrate (e.g., rock, gravel) which might otherwise wear hooves. However, hoof abnormalities are sometimes observed among young horses (~1 year of age) on Sable Island, while older horses (>15 years of age) can present a normal hoof phenotype.

Although we did not detect harmful concentrations of lathyrogens, genomic analysis would be necessary to conclude that Beach Pea plants are entirely incapable of BAPN synthesis. Similarly, we are also unable to reject the hypothesis that Beach Pea plants produce some other, yet unidentified disease-causing nonprotein amino acid. As an uncultivated plant species that is not commonly used as forage for livestock, a novel compound in Beach Pea plants is less likely to have been previously characterized. For example, while we tested for two of the most common lathyrism-causing compounds, Grass Pea has been found to produce the lathyrogen 2,4-diaminobutyric acid (DABA; Foster *et al.* 1987). The DABA inhibits ornithine decarboxylase and thereby prevents ammonia from entering the urea cycle (O'Neal *et al.* 1968), resulting in acute or chronic hyperammonemia (Rowe *et al.* 1993; Auron and Brophy 2012). The abnormalities in Sable Island horses that motivated our study are not consistent with acute hyperammonemia (Sharkey *et al.* 2006). However, Feral Horses living under challenging environmental conditions might be unlikely to survive to the point of acute presentation of this disease, and the symptoms of chronic hyperammonemia might be hard to detect using observational methods (Dunkel *et al.* 2011). In the future, Beach Pea plants should be assayed for DABA to provide a more comprehensive toxicological profile.

Author Contributions

Writing – Original Draft: R.J.G. and M.R.S.; Writing – Review & Editing: A.E., P.M.F.E., P.D.M., J.P., R.J.G., and M.R.S.; Conceptualization: P.D.M., J.P., R.J.G., and M.R.S.; Investigation: A.E., P.M.F.E., and R.J.G.; Methodology: A.E. and P.M.F.E; Formal Analysis: A.E. and P.M.F.E.; Funding Acquisition: P.D.M. and J.P.

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Supplementary Materials:

Video S1. Gait abnormality in a Sable Island Feral Horse (*Equus ferus caballus*), Sable Island, Nova Scotia, Canada. Video taken on 17 July 2021, by Mason R. Stothart.

Figure S1. Oxalyl-α,β-diaminopropionic acid spiking experiment.

Figure S2. β-aminopropionitrile spiking experiment.

Figure S3. Detection of β-aminopropionitrile (BAPN) in Beach Pea (*Lathyrus japonicus*) on Sable Island, Nova Scotia.